



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>A61K 38/20</b>		<b>A1</b>	(11) International Publication Number: <b>WO 95/10298</b>
			(43) International Publication Date: 20 April 1995 (20.04.95)
(21) International Application Number: PCT/US94/11588 (22) International Filing Date: 12 October 1994 (12.10.94) (30) Priority Data: 08/136,077                      12 October 1993 (12.10.93)                      US (60) Parent Application or Grant (63) Related by Continuation US    08/136,077 (CIP) Filed on                                      12 October 1993 (12.10.93)		(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>	
(71)(72) Applicants and Inventors: POLAN, Mary, Lake [US/US]; 425 Manuela Court, Palo Alto, CA 94306 (US). SIMON, Carlos [ES/ES]; Poeta Artola 12, 11a, E-46021 Valencia (ES). (74) Agent: DEHLINGER, Peter, J.; Dehlinger & Associates, P.O. Box 60850, Palo Alto, CA 94306-0850 (US).			
(54) Title: METHOD OF CONTRACEPTION			
(57) Abstract			
<p>Method of contraception utilizing IL-1 receptor antagonists are disclosed. In one embodiment, the method includes preventing ovulation and implantation of an embryo by administering to a subject a pharmacologically effective dose of an interleukin-1 (IL-1) receptor antagonist during the pre-implantation period. In a related method, the IL-1 receptor antagonist dislodges the implanted embryo, when it is administered during the post-implantation period. Also disclosed are contraceptive compositions, including IL-1 receptor antagonists, for use in the methods of the invention. Further disclosed is a method of selecting compounds for use in the contraceptive methods of the invention.</p>			

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

## METHOD OF CONTRACEPTION

### **Field of the Invention**

The present invention relates to methods and  
5 devices interfering with implantation of embryonic  
stage organisms in the maternal endometrium, and for  
preventing ovulation.

### **References**

- 10 Aarden, L.A., et al., *Eur. J. Immunol.* 17: 1411  
(1987).  
Ausubel, F. M., et al., Current Protocols in  
Molecular Biology, John Wiley and Sons, Inc., Media  
PA.
- 15 Barbas, C.F., et al., *Proc. Natl. Acad. Sci.  
USA* 89(10):4457 (1992).  
Chomczynski, P., *Biochemistry* 162:15 (1987).  
Clark, D.A., et al., *Ann N.Y. Acad. Sci.*  
626:524, (1991).
- 20 Cwirla, S.E. et al., *Proc. Natl. Acad. Sci. USA*  
87:6378-6382 (1990).  
Dinarello, C.A., R.C. Thompson., *Immunol Today*  
11:404 (1991).  
Dooley, C.T., et al., *Proc. Natl. Acad. Sci.*
- 25 *USA* 90(22):10822 (1993a).  
Dooley, C.T., et al., *Life Sci.* 52(18):1509  
(1993b).  
Dower, S.K., et al., *Nature* 324:266 (1986)  
Ecker, D.J., et al., *Nuc. Acids Res.* 21(8):1853  
30 (1993).  
Eichler, J., et al., *Biochemistry* 32(41):11035  
(1993).  
Eisenberg, S.P., et al. *Nature* 343:341 (1990).  
Fodor, S.P.A., et al., *Science* 251:767-773  
35 (1991).  
Furka, A., et al., *Int. J. Pept. Protein Res.*  
37:487-493 (1991).  
Granowitz, E.V., et al. *Cytokine* 4: 353-360  
(1992).

- Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual; Cold Spring Harbor laboratory, Cold Spring Harbor, New York.
- Haskill, S., et al., Proc. Natl. Acad. Sci. U.S.A., 88:3681-3688, (1991).
- Horuk, R., et al., Biochem. J. 260: 657 (1989).
- Houghten, R.A., Proc. Natl. Acad. Sci. USA 85:5131-5135 (1985).
- Houghten, R.A., et al., BioTechniques 4:522-528 (1986).
- Houghten, R.A., et al., Nature 354:84-86 (1991).
- Houghten, R.A., et al., BioTechniques 13:412-421 (1992).
- Irwin, J.C., et al., Fertil. Steril. 52: 761 (1989)
- Kenakin, T.P., Pharmacologic Analysis of Drug-Receptor Interaction, Raven Press, New York (1987).
- Kramer, A., et al., Pept. Res. 6(6):314 (1993).
- Kumar, et al., J. Controlled Release 18: 213-220 (1992).
- Maniatis, T., et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY (1982).
- McIntyre, B.K., et al., J. Exp. Med. 173:931 (1991).
- Mishell, B. B. and Shiigi, S. M., eds. (1980) Selected Methods in Cellular Immunology, W.H. Freeman and Co., San Francisco.
- Mizel, S.B., et al., J. Immunol. 138:2096 (1987).
- O'Gara, A. and Defrance, T.: Bioassays for interleukins. In Laboratory Methods in Immunology. H. Zola, Ed. CRC Press, 1990.
- Pinilla, C., et al., Biotechniques 13(6):901 (1992).
- Pinilla, C., et al., Gene 128(1):71 (1993).

- Polan, M., et al., *J. Clin. Endocrin. Metab.* 70:480 (1989).
- Romero, R., et al., *Amer. J. Obstet. Gynecol.* 167:1041-1045 (1992).
- 5        Schwartz, R.M. and Dayhoff, M.O. (1978) Atlas of Protein Sequence and Structure, 5 Suppl. 3: 353-358, Nat. biomed. Res. Fdn., Washington, D.C.
- Scott, J.K. and Smith, G.P., *Science* 249:386-390 (1990).
- 10       Sebestyen, F., et al., *Bioorg. Med. Chem. Lett.* 3:413-418 (1993).
- Seckinger, K., et al., *J. Immunol.* 139:1541-1545 (1987).
- Simon, C., et al., *Fertil. Steril.* 59: 791
- 15       (1993).
- Simon, C., et al., *J. CEM* (1993).
- Sims, J.E., et al. *Science* 241: 585 (1988).
- Taylor, G. et al. (1991) *Lancet* 337: 1411-1412.
- Zuckermann, R.N., et al., *Int. J. Pept. Protein*
- 20       *Res.* 40:498-507 (1992).

### Background of the Invention

The search for convenient and reliable means of birth control is one which occupies a significant

25       portion of the U.S. pharmaceutical industry. Public reluctance to accept abortion as a means of eliminating unwanted pregnancies has resulted in intensified efforts to provide alternative methods of post-coital birth control.

30       Currently the most widely used methods of birth control are those which prevent fertilization of the ovum. Known preventive methods currently in use include those which prevent conception by physical means (condoms), combined physical and chemical

35       barriers (e.g., diaphragms and spermicides) or estrogen-based contraceptive formulations. Prevention of pregnancy may also be effected by

insertion in the uterus of an intrauterine device (IUD), which may include a chemical component, such as the hormone progesterone. Although the foregoing methods of birth control are generally reliable when used correctly, they suffer from the disadvantage that a certain amount of planning must be taken on the part of at least one of the sexual partners prior to sexual intercourse.

As stated above, public attitudes in the U.S. have deterred widespread use of post-coital forms of birth control, or abortifacants. Mifepristone (RU-486) is an antiprogesterone which, when given during the first trimester of pregnancy, stimulates release of prostaglandins in the uterus, stimulates uterine motility and effects detachment from the uterus to cause abortion of the fetus. This compound, though an effective abortifacant, causes severe discomfort and may induce uterine bleeding requiring further medical management. Moreover, RU-486 it has not yet been approved for medical use in the United States.

Currently, there is only one formulation that is approved for prevention of pregnancy in those women who have had unprotected sexual intercourse. Diethylstilbestrol, the so-called "morning after pill", is a synthetic nonsteroidal compound having estrogen agonistic properties. This compound has the disadvantage that if gestation occurs, teratogenic effects such as vaginal adenocarcinoma and uterine malformations may occur in female fetuses. Also, a significant incidence of nausea and vomiting leads some patients to discontinue the medication in the middle of therapy.

#### Summary of the Invention

In one aspect, the invention includes a composition for use in preventing ovulation and

implantation of an embryo in a uterus of a mammalian subject, comprising an IL-1 receptor antagonist.

The interleukin-1 receptor antagonist may be a peptide, such as interleukin receptor antagonist IL-1ra (SEQ ID NO: 2) or icIL-1ra (SEQ ID NO: 4), or an oligopeptide selected from a combinatorial library. In the latter case, the compound is selected from a combinatorial library of compounds, by the steps of measuring (i) agonist activity, and (ii) antagonist activity of a library of compounds, in a functional IL-1 receptor assay, and selecting a specific compound in the library if it has (i) substantially no agonist activity, and (ii) substantial antagonist activity.

Alternatively, as compound may be selected by (i) reacting compounds from a library with cells having an IL-1 cell-surface receptor, in the presence of a reporter-labeled IL-1, (ii) assaying the ability of library compounds to displace labeled IL-1 from such cell-surface receptors, and (iii) selecting a compound from the library effective to displace labeled IL-1 from the cells.

Also forming part of the invention is a contraceptive device for use in preventing ovulation and implantation of an embryo or in dislodging an embryo in a mammalian uterus. The device includes a vaginal insert or a cervical cap, and compound release means in said insert or cap for releasing an IL-1 receptor antagonist compound at a dose effective to prevent or disrupt implantation of an embryo in the uterus.

In another aspect, the invention includes a method of identifying a compound for use in preventing ovulation and implantation of an embryo or in dislodging an embryo in a mammalian uterus. The method includes measuring (i) agonist activity, and (ii) antagonist activity of a library of

compounds, in a functional IL-1 receptor assay, and selecting a specific compound in the library if it has (i) substantially no agonist activity, and (ii) substantial antagonist activity.

5       The method may further include measuring further includes measuring the binding affinity of the compound in an IL-1R tI IL-1 ligand competitive displacement binding assay, and said selecting further includes selecting the compound if its  
10       affinity in such binding assay is at least within two orders of magnitude of the binding affinity of an IL-1 receptor antagonist selected from the group consisting of IL-1ra and icIL-1ra.

15       IL-receptor antagonist compounds used in the methods and compositions described herein have several advantages over known contraceptive methods. They act locally, rather than systemically, resulting in few if any systemic side effects. Moreover, they are effective after the fertilization  
20       event and can be used as an alternative to currently practiced post-fertilization means of terminating pregnancy.

#### **Brief Description of the Figures**

25       Figures 1A and 1B show Northern blots (1A) of IL-1R tI mRNA in cultured human endometrial stromal cells (ESC), where the migration positions of 18S and 28S ribosomal RNA markers are indicated to the left, and corresponding quantitation of the blots by  
30       densitometric analysis (1B);

      Figures 2A and 2B show Northern blots (2A) of IL-1R TI MRNA in cultured human endometrial epithelial cells (EEC), where marker positions are indicated as in Figure 1A, and show corresponding  
35       quantitation of the blots by densitometric analysis (2B);



Figure 3 (A-E) shows indirect immunofluorescence immunolocalization of mouse IL-1R TI in mouse uterus (3A-3D) and of IL-1 $\beta$  (3E) in the mouse embryo;

5        Figure 4 (A-E) shows micrographs of sections of mouse uteri taken at various post-fertilization stages of untreated mice (4A, 4C, 4E) and of mice treated with hr IL-1ra (4B, 4D, 4F);

10        Figure 5 is a temporal depiction of the human female menstrual cycle, indicating appropriate times in the cycle at which intervention with IL-1ra receptor blockade will prevent embryonic implantation; and

15        Figure 6 shows DNA and amino acid sequences of IL-1ra as SEQ ID NO: 1 and SEQ ID NO: 2, respectively; and

Figure 7 shows the DNA and amino acid sequences of iCIL-1ra as SEQ ID NO: 3 and SEQ ID NO: 4, respectively.

20

## **Detailed Description of the Invention**

### **I. Definitions**

25        The term "endometrium", as used herein refers to the glandular inner layer of the uterus, overlying a muscular myometrial layer.

      The term "endometrial stromal cell" (abbreviated "ESC") refers to a specific endometrial cellular component underlying epithelial cells in the uterus.

30        The term "endometrial epithelial cells" (abbreviated "EEC"), refers to a specific cellular component of the uterine endometrium. These cells are in contact with the lumen of the uterus.

35        The term "oocyte" refers to the immature female egg cell present in the ovaries and having a full diploid complement of chromosomes.

The term "ovum" refers to the mature female haploid egg cell which has matured from an oocyte by undergoing meiosis. The ovum is released at the time of ovulation from the follicles of the ovaries.

5       The term "ovulation" refers to the release by the follicles of one or more ova. Ovulation is characterized in humans by specific hormonal changes; in particular, it occurs just subsequent to a spike in luteinizing hormone (LH) and follicle  
10 stimulating hormone (FSH) levels and a decrease in level of estrogen.

The term "embryo" refers to a fertilized ovum.

The term "blastocyst" refers to a specific stage of embryo, characterized by the presence of a  
15 central cavity surrounded by dividing cells.

The term "peri-implantation" period, refers to a time period shortly before, during and after uterine implantation of the embryo, as described in Section V herein.

20       The term "IL-1 receptor antagonist" refers to a compound that blocks the effects of endogenous or exogenously added IL-1. IL-1 receptor antagonists may act competitively or non-competitively at the receptor level.

25       The term "IL-1 inhibitor" refers to a compound that blocks IL-1 activity. IL-1 inhibitors include IL-1 receptor antagonists, but also include agents that act by decreasing an amount of endogenous IL-1 available or released.

30

## II. Interleukin-1 Receptor Antagonists

Interleukin-1 receptor antagonist compounds are characterized by an ability to inhibit binding and biological effects of IL-1 $\alpha$  and IL-1 $\beta$ , as described  
35 in Section III, below. Receptor antagonist compounds include, in addition to competitive inhibitors of receptor binding, other

macromolecules, such as antibodies or exogenously added IL-1 binding proteins that interfere with IL-1 binding to its receptor.

IL-1 receptor antagonist compounds that are particularly useful in practicing the present invention are those which interfere with the interaction between IL-1 and its receptor(s) in the uterus. Particularly useful compounds include IL-1ra, a specific peptide antagonist and derivatives thereof, collectively termed IL-1ra peptides. It is the discovery of the present invention that blocking the interaction between embryonic IL-1 and uterine IL-1 receptors, particularly type I receptors, in the mammalian uterus results in disruption of implantation of the embryo in the uterus.

#### A. IL-1 Receptor Antagonists

Several naturally occurring IL-1 receptor antagonist compounds have been identified and isolated from biological sources including serum, synovial exudates, and urine. Some of these compounds are not specific to the IL-1 receptor, since they also inhibit binding and/or activity of other cytokines, notably IL-2. While such non-specific inhibitors may be used in practicing the methods of the invention described herein, antagonist compounds that are specific to the IL-1 receptor, and particularly the IL-1 type I receptor (IL-1R TI) are generally preferred for use in the invention.

#### B. IL-1ra peptides

IL-1ra is IL-1 receptor antagonist that can be used in practicing the invention. IL-1ra is a 22-25 kDA peptide which is found in the urine of febrile human patients suffering from monocytic leukemia (Seckinger), but has also been identified in other tissues, including human monocytes (Eisenberg).

This compound is an antagonist of binding of IL-1 $\alpha$  and IL-1 $\beta$  at the IL-1 receptor, particularly at the IL-1 Type I receptor.

As is described in Section III, below, IL-1ra  
5 selectively inhibits binding of IL-1 ( $\alpha$  or  $\beta$ ) to the IL-1 receptor. The selectivity of this compound is evidenced by the fact that it does not inhibit biological effects of IL-2 or other cytokines.

IL-1ra cDNA has been identified and cloned from  
10 a monocyte library (Eisenberg). The complete DNA sequence is shown in Figure 7 as SEQ ID NO: 1. Based on the cDNA sequence, an amino acid sequence has been deduced and verified. This sequence is shown in Figure 7 as SEQ ID NO: 2. The cDNA shown  
15 encodes a 25 amino acid secretory peptide leader sequence followed by a 152 amino acid polypeptide.

For use in the present invention, IL-1ra is generally isolated from natural sources or produced by recombinant methods known in the art (Seckinger;  
20 Ausubel) and described in the sections which follow.

Derived from the same gene, but having an alternate splicing pattern, is an intracellular form of IL-1ra, termed icIL-1ra (Haskill). This IL-1ra peptide lacks the N-terminal 21 amino acids the 25  
25 residue signal sequence of IL-1ra. In their place are three different amino acids. The DNA and peptide sequences are shown as SEQ ID NO: 3 and SEQ ID NO: 4, respectively, in Figure 7.

### 30 1. Isolation of IL-1ra peptides

#### a. Purification of IL-1ra from Biological Sources.

IL-1ra has been isolated from a number of biological sources, using standard biochemical  
35 purification procedures known in the art. One such procedure that has been published in the medical literature by Carter, et al. involves isolation of the peptide from culture supernatants of

differentiated U937 (myelomonocytic) cells. This method includes ultrafiltration through molecular weight cut-off filters, Superose 12 FPLC, followed by two rounds of TSK Biosil 125 HPLC and C4 RP HPLC. (Carter). This method, summarized in Example 6) can be used to obtain IL-1ra of sufficient purity for use in the inventive methods described herein. Alternatively, the purification method described by Hannum et al., incorporated herein by reference, can be used to purify IL-1ra for use in the methods and compositions of the present invention.

b . Recombinant Production of IL-1ra.

Human recombinant IL-1ra (hr IL-1ra) can be expressed in bacterial or mammalian expression systems, according to methods known in the art. In one such method, used to produce IL-1ra for the studies reported herein, and detailed in Example 5, IL-1ra is expressed as a fusion protein using a pMAL vector (New England Biolabs, Beverly, MA). This vector is constructed to contain the DNA clone shown as SEQ ID NO: 1 in Figure 6. Alternatively or in addition, the method described by Hannum et al. in U.S. Patent No. 5,075,222, incorporated herein by reference, is used to produce IL-1ra

The expressed fusion protein is purified from the periplasm of E. coli by Amylose affinity chromatography (New England Biolabs). The isolated fusion protein is cleaved by Factor Xa treatment to produce hr IL-1ra and the carrier maltose binding protein. Homogeneity of hIL-1ra is achieved with further purification using, for example, Q-sepharose and Sephacryl S-300 gel filtration chromatographies (Pharmacia), according to methods known in the art. The purified protein has a molecular mass (Mr) of 17,000. Specific activity of recombinant IL-1ra can be measured by any of a number of biological assays for

ability to block IL-1 activity. One such assay is a mouse D10 cell proliferation assay (Polan, et al.), detailed in Example 8 and described in Section III below.

5

## 2. Isolation of icIL-1ra

The intracellular form of IL-1ra (icIL-1ra) is produced by recombinant expression methods, such as the COS cell expression method described in the reference by Haskill, et al. (incorporated herein by reference). According to this method, COS cells are transfected with a SR/ $\alpha$  vector containing a *Pst* I fragment containing the sequence shown in Figure 7 as SEQ ID NO: 3. Cell lysates are then used for purification of the icIL-1ra peptide, essentially as described for IL-1ra, above.

10

15

### C. Derivatives of IL-1ra Peptides

In accordance with known principles of conservative amino acid substitutions in a protein, it can be appreciated that derivatives of IL-1ra can be constructed by making conservative amino acid substitutions into the parent IL-1ra primary structure SEQ ID NO: 2, or into the parent icIL-1ra structure SEQ ID NO: 4. Such conservative amino acid substitutions are known in the art to encompass such substitutions as serine for alanine and the like. For example, according to a standard Dayhoff frequency exchange matrix, twenty naturally occurring amino acids are placed in six categories, based on substitutions in nature (Schwartz). Conservative substitutions can be made between amino acids present in any single class. According to this scheme, the six classes are as follows: Class I, CYS; Class II, Ser, Thr, Ala, Gly, representing small aliphatic side chains and OH group side chains; Class III, Asn, Asp, Glu, Gln, representing

20

25

30

35

neutral and negatively charged side chains capable of forming hydrogen bonds; Class IV, His, Arg, Lys, representing basic polar side chains; Class V, Ile, Val, Leu, representing branched aliphatic side chains, and Met; Class VI, Phe, Tyr, Trp, representing aromatic side chains. Proline and 4-hydroxyproline are members of Class II; however, substitution of these amino acids for other Class II residues may introduce new secondary structure into the polypeptide backbone. Proline and 4-hydroxyproline may be considered to comprise an additional class, for purposes of conservative substitution. Furthermore, each class may include certain related amino acid analogs, such as ornithine, homoarginine, N-methyl lysine, dimethyl lysine (Class IV) and halogenated tyrosine in Group VI.

IL-1ra derivatives suitable for use in the present invention will have an IL-1 antagonist profile that is substantially like that exhibited by IL-1ra, as described in Section III, below. That is such derivatives will have very little or no IL-1 agonist activity and will have activity profiles that approximate those of IL-1ra. By "approximate" is meant that such compounds will have a potency in an IL-1 antagonist assay which is minimally within about 2-3 orders of magnitude of the potency of IL-1ra or icIL-1ra.

Thus in selecting as IL-1 receptor antagonist compounds for use in the invention IL-1ra derivatives as described above, a practitioner will be guided by the present invention to construct such derivatives according to the conservative substitution guidelines discussed above using methods known in the art, such as by site-specific oligonucleotide-directed mutagenesis techniques (Ausubel et al.; Maniatis et al.). Available from

commercial sources are kits for site-directed mutagenesis (Stratagene, La Jolla, CA), that can be used in constructing IL-1ra peptide derivatives.

5 In accordance with the invention, IL-1ra peptide derivatives formed according to one or more of the above techniques are then tested for binding to the IL-1 receptor (IL-1R tI) and/or for inhibition of IL-1 activity in vitro, according to the methods set forth in Section III, below.

10 Derivative compounds having binding and inhibitory activities, or minimally, functional inhibitory activity, approximating a range defined by the activity of IL-1ra and icIL-1ra, described below, are also utilizable in the methods and compositions

15 of the present invention.

It is also appreciated that in accordance with the discovery of the present invention interference with uterine implantation may also be accomplished by other compounds that diminish the biological

20 effects of IL-1 in vivo, such as by reducing production of IL-1 by the embryo or by stimulating endogenous production of IL-1ra. Thus, a compound such as zymosan (Tenidap®), that reduces secretion of endogenous IL-1, may also find use in the present

25 invention.

#### D. Monoclonal antibodies against IL-1 Receptor

Monoclonal antibodies raised against the mouse

30 type I IL-1 receptor have been shown to block immune and inflammatory responses of IL-1 at the receptor (McIntyre). In accordance with the present invention, monoclonal antibodies having such binding and inhibitory properties with respect to the human

35 IL-1R tI, can be used in the methods and compositions of the present invention.

Preferably, monoclonal antibodies will be raised against the human IL-1R tI, according to



methods well known in the art (Mishell). Immunogen material for preparing such antibodies will be prepared by biochemical or recombinant techniques as described in Section II, then used to immunize a suitable host animal, including, but not restricted to, mouse, rat, goat, or rabbit. Mice are generally considered preferable for such purposes.

Host animals are inoculated with an appropriate amount of a pure or partially pure human IL-1R tI. The amount will depend on the animal used. For mice, an amount of immunogen corresponding to about 1-100  $\mu$ g protein will be sufficient for such purposes. Sera are then tested for presence of antibodies, such as in an ELISA test according to standard methods known in the art. Spleens are then removed from animals exhibiting evidence of immunoreactivity with human IL-1R tI. Dispersed spleen cells are then fused with an appropriate fusion partner myeloma cell, using hybridoma production techniques known in the art (Harlow). Cultures are further tested for presence of immunoglobulin and anti-IL-1R tI activities by ELISA and/or receptor binding assays, such as the receptor binding assay described herein. Preferably, for use in humans, such antibodies will be "humanized" according to recombinant methods. For example, according to one such method, mouse-derived monoclonal antibody variable regions are combined with human constant regions to produce "humanized" mouse monoclonal antibodies (Taylor). Such humanized antibodies may be preferred for use in humans, due to their reduced potential for production of non-specific immune reactions.

### III. Method of Screening for IL-1 Receptor Antagonists

In accordance with the discoveries described herein, it can be appreciated that the present

invention defines a method for selecting or screening compounds for use as contraceptives. In this regard, compounds having IL-1 receptor antagonism activity that is substantially similar to  
5 such activity exhibited by IL-1ra will be useful in the methods and compositions of the invention.

It can be further appreciated that candidate compounds include, but are not limited to, IL-1ra peptide derivative compounds, described above.  
10 Other preferred candidate compounds include peptide fragments of IL-1ra, as well peptides and other compounds generated by combinatorial libraries, as described below, or a random-sequence peptide library, such as a library using filamentous phage  
15 fUSE5 as a vector (Scott; Cwirla).

#### A. Combinatorial Libraries

A variety of combinatorial libraries of random-sequence oligonucleotides, polypeptides, or  
20 synthetic oligomers have been proposed (Kramer; Houghten, 1985, 1986, 1991, 1992; Ohlmeyer; Dooley, 1993a-1993b; Eichler; Pinella, 1992, 1993; Ecker; and Barbas). A number of small-molecule libraries have also been developed (e.g., Ellman).

25 Combinatorial libraries of oligomers may be formed by a variety of solution-phase or solid-phase methods in which mixtures of different subunits are added stepwise to growing oligomers or parent compound, until a desired oligomer size is reached.  
30 A library of increasing complexity can be formed in this manner, for example, by pooling multiple choices of reagents with each additional subunit step (Houghten, 1991).

Alternatively, the library may be formed by  
35 solid-phase synthetic methods in which beads containing different-sequence oligomers that form the library are alternately mixed and separated,

with one of a selected number of subunits being added to each group of separated beads at each step (Furka, 1991; Lam, 1991, 1993; Zuckermann; Sebestyen).

5        Still another approach that has been proposed involves the synthesis of a combinatorial library on spatially segregated arrays (Fodor).

      In one preferred embodiment, the library is a combinatorial library of hexapeptides or  
10    heptapeptides, containing all or some defined subset of permutations of amino acids. The approach generally follows published method (Houghten, 1991).

      One screening assay for identifying IL-1 receptor antagonists from a combinatorial library is  
15    based on inhibiting IL-1 activity in a physiological setting. Such activities can be assessed in certain in vitro assays known in the art. Described below are representative in vitro assays useful in measuring IL-1 activity and blockade thereof. It is  
20    appreciated that any of a number of known IL-1 activity assays can be used to assess inhibition of IL-1. Such assays may be substituted for the particular assays described below without deviating from the form of the present invention.

25        Alternatively, or in addition, IL-1 receptor antagonist compounds in a combinatorial library can be identified on the basis of their ability to displace IL-1 from its normal receptor sites on a cell surface. For example, the library compounds  
30    may screened for their ability to displace reporter-labeled, e.g., radiolabeled, IL-1 $\alpha$  or IL-2 $\beta$  from the surface of cells having a suitable IL-1 surface receptor, such as the EL-4 cells described in Example 7. The assay generally follows the  
35    procedure disclosed in this example, for displacement of IL-1 $\alpha$  or IL-1 $\beta$ .

The identity of library compounds with observed inhibitory and/or binding displacement activity can be determined by conventional means, such as iterative synthesis methods in which sublibraries  
5 containing known residues in one subunit position only are identified as containing active compounds.

B. Binding to IL-1R tI

10 Although IL-1 receptor antagonists that are useful in the present invention are not necessarily competitive antagonists of IL-1 at the IL-1R tI, compounds, such as IL-1ra, which bind competitively are preferred in at least one embodiment of the  
15 invention. A determination that binding is competitive, as opposed to non-competitive, may be assessed using a number of pharmacological tools, including, for example, Schild plot regression analysis of dose-response curves generated from  
20 functional assays (Kenakin).

One assay for determining whether a compound binds competitively to an IL-1 receptor, is a competitive displacement binding assay (Mizel). In this assay, as in the one detailed in Example 7,  
25 intact cells are incubated with  $^{125}\text{I}$ -IL-1 $\alpha$ , and binding to the cells is determined following separation from the cells of unbound  $^{125}\text{I}$ -IL-1 $\alpha$ . In such an assay, competitive binding is evidenced by competitive displacement of bound  $^{125}\text{I}$ -IL-1 $\alpha$  from the  
30 cells when the cells are incubated with a test binding compound.

According to results of competitive displacement assays, such as that reported above, IL-1ra has approximately the same affinity as does  
35 IL-1 in binding to EL-4 cells, as well to 3T3 (fibroblasts) and CHO cells (McIntyre). IL-1 receptor antagonists preferred for use in the

present invention will have affinities for the IL-1 binding site, and more specifically, for IL-1R tI, which are substantially that of IL-1ra for the IL-1Rti. That is, preferred antagonists will exhibit  
5 affinities that are higher than or only slightly lower than ( $K_i$  within about 2-3 log units) the affinity of IL-1ra for IL-1R tI, when compared in the same binding assay. In one cell binding assay that employs YTNC1 cells, IL-1ra had a  $K_i$  of about  
10 0.25 nM. Accordingly, when measured in this system, it is anticipated that a receptor antagonist preferred for use in the present invention will have a  $K_i$  that is at least about  $10^{-7}$  M.

It is also appreciated that activity of a  
15 compound in a competitive displacement binding assay does not generally predict whether the test compound is an agonist or antagonist. Therefore, competitive displacement activity is not generally considered definitive of competitive antagonism. Compounds  
20 shown to bind competitively to the receptor are tested in one or more functional assays, such as one or more of those described below, to determine whether binding to the receptor results in agonist or antagonist activity at the receptor.

25

#### C. Functional Assays for IL-1 Receptor Antagonism

Functional or biological assays provide means of assessing whether a compound has agonist or antagonist activity at the IL-1R tI. Such  
30 assessment is generally carried out by measuring activity of a known agonist compound in the assay, and comparing to this activity the activity of the agonist in the presence of test compound. In addition, the test compound is also evaluated in the  
35 functional assay in the absence of the standard known agonist, to determine whether it possesses agonist activity as well. In general, preferred

antagonists will exhibit little or no agonist activity, relative to antagonist activity. Moreover, their antagonistic activity will be substantially similar to that of IL-1ra, as  
5 discussed below.

#### 1. Mouse D10 cellular Proliferation Assay

Example 8 details a method for assessing IL-1 receptor antagonist activity in an assay in which  
10 IL-1 stimulates proliferation of mouse T-helper cells. Human IL-1 $\alpha$  and human IL-1 $\beta$  stimulate proliferation of cells, measured by uptake of tritiated thymidine, in such an assay. In this assay, 30 nM IL-1ra was effective to completely  
15 inhibit the effects of IL-1 $\alpha$  or IL-1 $\beta$  when the agonists were present at concentrations ranging from about 0.1 pM to approximately 50 nM.

More generally, to assess relative antagonist potency in such an assay, IL-1 $\alpha$  or IL-1 $\beta$  is added to  
20 the assay at a half-maximal stimulatory concentration (EC50). The concentration of IL-1ra or test compound is varied from about  $10^{-12}$ M to  $10^{-6}$ M, to determine a  $K_i$  value for the compound in the assay, according to standard procedures (Kenakin).  
25 Compounds preferred for use in the invention will have a  $K_i$  that is substantially the same as a  $K_i$  determined for IL-1ra or icIL-1ra in the assay. That is, they will have a  $K_i$  that is less than about 2-3 log units higher than the  $K_i$  determined for IL-  
30 1ra or icIL-1ra in the assay.

#### 2. Inhibition of serum IL-6 production

A pharmacological effect of IL-1 is stimulation of IL-6 production in serum. IL-1ra inhibits such  
35 production, when given to mice in addition to IL-1 (McIntyre). Example 9 details a method by which IL-1 receptor antagonism is assessed in mice according

to the ability of a compound to block such stimulation. In this assay, as in the proliferation assay, it is anticipated that preferred IL-1 antagonist compounds will behave substantially like  
5 IL-1ra or icIL-1ra, as described for the proliferation assay in sub-part 1 above.

#### IV. IL-1 Receptor Antagonist-mediated Contraception

##### 10 A. Involvement of IL-1 in Intrauterine Function

Certain paracrine cytokines are known to be involved in interactions between the blastocyst and endometrium. For example, secretion by the maternal uterus of the leukemia inhibitory factor (LIF) has  
15 been demonstrated to be required for successful implantation of the fertilized ovum. Other paracrine cytokines which have been implicated in implantation include maternal colony stimulating factor-1 (CSF-1), mutations of which have been shown  
20 to compromise implantation. It is a discovery of the present invention that disruption (prevention or dislodgement) of embryonic implantation can be effected *in vivo* by blockade in the maternal endometrium of the endogenous cytokine IL-1 receptor  
25 type I (IL-1R tI).

The interleukin-1 (IL-1) system includes IL-1 $\alpha$ , IL-1 $\beta$ , IL-1 receptor antagonist (IL-1ra) and the IL-1 receptor (IL-1 R). Two IL-1 receptor subtypes have been identified. They are termed IL-1R type  
30 I (IL-1R tI (Sims)) and IL-1R type II (IL-1R tII (Horuk)). Both IL-1 $\alpha$  and IL-1 $\beta$  bind to IL-1R tI (Dower), and their effect on this receptor is antagonized by IL-1ra.

IL-1 and its receptor have both been localized  
35 to endometrial cells in mice and humans. In addition, IL-1 $\alpha$  and IL-1 $\beta$  have been shown to be secreted by the human embryo prior to implantation.

The IL-1 receptor (IL-1R tI) is also expressed in

the human endometrium throughout the menstrual cycle. The agonist IL-1 has been used to induce pre-term parturition in mice, and the antagonist IL-1ra inhibits this abortifacant effect of IL-1 (Romero). In contrast to the anti-abortive effects of IL-1ra described above, as described in the following sections, an important aspect of the present invention is the discovery that antagonism of IL-1r tI by receptor antagonists is effective to prevent implantation of a fertilized ovum, when IL-1ra is present in the uterus during the implantation event, as well as to dislodge such implantation after it has occurred. More generally, according to these findings, it can be stated that IL-1 receptor antagonism is effective to provide contraception during the peri-implantation period. This period is a time during which the fate of the embryo can be manipulated by hormonal or biochemical means related to the implantation process. In the context of the present invention, this time period refers to the time period during which administration of IL-1ra to the subject can disrupt, that is, prevent or reverse implantation, as described in Section V below.

Additionally, studies carried out in support of the present invention indicate that IL-1 receptor antagonism is effective to prevent ovulation in animals stimulated to ovulate, as described in subsection D, below.

30

B. Embryonic Regulation of Expression of IL-1 Receptor in Uterine Endothelial Cells

Experiments carried out in support of the present invention show that fertilized ova secrete substances into their surroundings which, when brought into contact with uterine endometrial cells, stimulate the cells to express increased levels of the IL-1 receptor type I (IL-1R tI).



Figures 1 and 2 show results of experiments in which human uterine endometrial stromal cells (ESC) and endometrial epithelial cells (EEC), isolated from ovulatory women in the luteal phase, were cultured and grown in basal conditions in steroid-free media, as detailed in Example 1. The resulting monolayers were maintained for 6 days under the growth conditions prescribed by an established model for *in vitro* decidualization (Irwin), as detailed in Example 1. At that time, one of the following embryonic products was added to the endometrial culture medium: human recombinant IL-1b (rIL-1b), Human Chorionic Gonadotropin (HCG), Platelet activation factor (PAF), or conditioned media from human blastocyst (HBCM; prepared as described in Example 1). Cells were subjected to RNA analysis by Northern blot with human IL-1R tI cRNA probe, according to established procedures, as described in Example 1. For quantitation of results, Northern blot data were normalized to a standard 28S rRNA cDNA probe standard, according to established methods (Simon).

As shown in Figures 1 and 2, IL-1R tI mRNA and immunoreactive IL-1R tI were found in both human ESC and EEC. In human ESC, cultured under basal conditions in the presence of progesterone (P) and epidermal growth factor (EGF) only IL-1R tI mRNA expression was upregulated 2.6-fold after 8 days in culture. Addition of embryonic products to the cells for 2 days (from day 6 to 8) produced a further up-regulation of IL-1R tI as follows: conditioned media from human blastocyst (7.5-fold increase), PAF (5.7-fold increase), IL-1b (4.2-fold increase), hCG (3.5-fold increase) (Figures 1A and 1B).

In EEC, IL-1R tI mRNA constitutively expressed at higher at levels than those observed in ESCa.

However, in these cells, IL-1R tI mRNA was not significantly regulated by any of the embryonic products observed to increase receptor expression in ESC (Figures 2A and 2B).

5

### C. Preventing Embryonic Implantation

#### 1. Murine Model of Embryonic Implantation.

A mouse model was used to study implantation. The validity of the mouse model to the study of implantation in humans was assessed by determining (a) the presence in the mouse uterus endometrium of immunoreactive IL-1R tI following pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) administration, and (b) the presence of IL-1R tI in the epithelial region surrounding the blastocyst during the peri-implantation period, specifically at day 4 of pregnancy, since these conditions have been observed in human uterine tissues. Other indicators of the validity of the murine model to human conditions are known in the art (Clark). Specifically, IL-1 $\alpha$  and IL-1 $\beta$  mRNAs and IL-1 bioactivity in the peri-implantation uterus are known to increase from day 3 and peak between days 4 and 5, post-fertilization. Blastocyst implantation generally occurs late on post-fertilization day 4 in mice, but may be delayed until about day 8, due to the phenomenon of "delayed implantation" which is apparently inherent to mice.

Figure 3 shows the results of experiments in which immunoreactivity of IL-1R tI and IL-1 $\beta$  were measured in the mouse maternal endometrium and in the mouse placenta, respectively, following fertilization, as detailed in Example 2. As shown, mouse IL-1R tI is localized in the apical region of the luminal epithelium and in scattered areas of the stroma (Figures 3A and 3B). Increased IL-1R tI

immunoreactivity was observed in the epithelial tissue surrounding the blastocyst, as indicated by the arrow in Figure 3D. As shown in Figure 3E, at day 9 of pregnancy, immunoreactive IL-1 $\beta$  was present in the mouse placenta (p) with increased staining at the interface with the maternal endometrium (m). These results serve as biochemical validation of the mouse model for human implantation, with respect to the IL-1 cytokine system, as described above.

Although gestational length differs considerably between mouse and human, since certain common developmental stages can be identified, comparable timepoints can be established between the two species. Extrapolation of critical gestational timepoints from mice to humans is known in the art to be within the skill of the practitioner; however, when appropriate, such extrapolations are also provided herein.

## 2. Time Course of Embryonic Implantation

For the purposes of the present discussion, the female reproductive system comprises the ovaries, Fallopian tubes or oviducts, uterus and vagina. Ova mature in the ovarian follicles from oocytes contained within. Mouse and humans ovulate (release mature ova from the follicles) in response to approximately the same hormonal stimuli, which include a periodic spike of luteinizing hormone (LH) produced in the pituitary and measurable in the serum. Ovulation in mice occurs spontaneously about every 4 days; in humans, ovulation occurs about every 28 days.

Following ovulation, the ovum is taken into an oviduct, where it is thought to remain fertile for 10-24 hours in humans and for about 12 hours in mice. Fertilization, if it occurs, generally occurs

in the ampulla of the fallopian tube in humans, also known as the oviduct in mice.

In the mouse, following the release of sperm into the female reproductive tract, the sperm  
5 undergo capacitation, a process that renders them competent for fertilization. Capacitation usually occurs about 1 hour following ejaculation. Mouse sperm generally remain viable in the female reproductive tract for about 6 hours after  
10 ejaculation. Human sperm generally remain viable for at least about 48 hours after ejaculation. Generally, according to these timepoints indicate it is thought that for fertilization to occur in humans, coitus should occur no more than about 48  
15 hours before or 24 hours after ovulation.

Following fertilization, the maturing embryo migrates to the uterus. During this migration, the embryo undergoes maturation through cell division. By the time the embryo reaches the uterus, generally  
20 about 2.5-3 days post-fertilization in mice and about 4 days post-fertilization in humans, it has progressed through the morula stage to the blastocyst stage. At the uterus, the embryo, generally in morula or blastocyst stage, attaches to  
25 the uterine wall in a process known as implantation. In humans, implantation occurs between days about 4 to 7, and particularly 5.5 to 6 after ovulation. In mice, implantation usually takes place between day 4.5 and day 5 following ovulation, though it can be  
30 delayed until about day 8, due to the delayed implantation phenomenon discussed above.

### 3. Inhibition of Embryonic Implantation by Blockade of IL-1 Receptors

35 It is one discovery of the present invention that blockade of the IL-1 receptor can prevent embryonic implantation, thereby interfering with

pregnancy. In experiments carried out in support of the invention, IL-1ra was administered by intraperitoneal injection to female mice previously mated with males of the same strain and age, and outcome was assessed by measuring the number of successful implantations, as detailed in Example 3. Briefly, 12-week-old B6C3F-1 female mice were super-stimulated to ovulate by administration PMSG/hCG. Stimulated females were mated with males of the same strain and age. Coitus was assessed by detection of vaginal sperm plug 24 hours after hCG injection. At day 3 of pregnancy, mice were divided in 3 groups: control non-infected, control buffer injected, and mice injected with IL-1ra.

Results of the mouse implantation studies are shown in Table 1. As indicated, treatment of female mice with IL-1ra resulted in a significant decrease in the pregnancy rate ( $p=.000115$  compared to control buffer injected and non-injected animals (Fisher exact test). These results indicate that post-fertilization administration of IL-1ra is effective to interfere with pregnancy.

Table 1

Effect of rh IL-1ra Administration on the Reproductive Outcome of Pregnant Mice

Control Group Non-Injected (n = 17)	Control Group Buffer Injected (n = 19)	IL-1ra Injected Group (n = 15)
Pregnant = 10 (58.8%)	Pregnant = 14 (73.3%)	Pregnant = 1 (6.7%)
Non Pregnant = 7 (41.2%)	Non Pregnant = 5 (26.3%)	Non Pregnant = 14 (93.3%)

In further support of the invention, a morphologic longitudinal study was carried out to monitor embryonic implantation in the mouse from pregnancy day 4 to day 9 in control, untreated

animals compared with hr IL-1ra treated animals, as described above. Fig. 4 shows microscopic sections of mice uteri made at various days post-fertilization. With reference to Figure 4A, showing a longitudinal section of the uterus at day 4. Here, the free blastocyst is located within the lumen of the uterus. With reference to Figure 4C and Figure 4F, showing sections taken at days 7 and 9 of normal pregnancy, respectively, the mouse embryo is observed to be completely implanted in the stroma.

In the IL-1ra treated animals, the free blastocyst was also found within the uterus by post-fertilization day 4, shown in Figure 4B. By day 7 the embryo in the IL-1ra treated animal had neither attached nor implanted (Fig 4D). At day 9, no stromally implanted embryos were observed in the IL-1ra treated animal; however degenerated trophoblast cells were found in the uterine lumen (Figure 4F).

The studies described above indicate that embryonic IL-1 binding to maternal IL-1R tI is required for successful implantation of the embryo in the uterus, since a specific antagonist of this binding prevents the implantation process. These studies also provide basis for the contention that plentiful distribution of IL-1R tI throughout the luminal epithelium is required for initiation of receptor-ligand interaction initiated wherever the embryo attaches. Once the embryo has traversed the epithelium and begins stromal invasion, its own secretion of IL-1, and perhaps other paracrine factors, induces IL-1R tI in the surrounding stroma, allowing implantation to proceed. However, as shown herein, blockage of the IL-1R tI by an antagonist is sufficient to prevent implantation.

Evidence that the effects observed in the mouse model were specific effects, rather than non-

specific toxic effects on the embryo was provided by experiments in which early (2-cell) mouse embryos were flushed from the oviducts of the same group of mice used for *in vivo* experiments. The embryos were  
5 incubated with increasing concentrations of rh IL-1ra for three days, as detailed in Example 4A. Assessment of viability was made, based on the number of embryos that reached blastocyst stage after 72 hours in culture. As indicated by the  
10 results presented in Example 4, no significant differences in survival were noted between embryos incubated in the presence of concentrations of IL-1ra ranging from 0 to 200 µg/ml.

Recombinant human IL-1ra was further tested for  
15 possible effects on later developmental stages of the mouse embryo, including blastocyst hatching, trophoblast outgrowth and migration, as detailed in Example 4B. Blastocysts were cultured 5 days on fibronectin-coated plates in the presence of absence  
20 of IL-1ra, as described in Example 4B. Hatching, outgrowth and migration were found to be similar in the presence or absence of rh IL-1ra. These experiments demonstrate that rh IL-1ra is not toxic to the embryo itself and provide further evidence  
25 that the contraceptive effects of IL-1ra are specific to the implantation process.

#### D. Dislodging Implanted Embryos

It is also an observation of the present  
30 invention that interference with implantation can occur after the initial implantation event. To be effective, an IL-1R tI antagonist compound will preferably be administered during the post-implantation period, which extends from the  
35 implantation period to at least several days following the initial implantation event, as indicated in Figure 5.

#### E. Prevention of Ovulation

A further observation of the present invention is that IL-1 receptor antagonism is effective to inhibit or prevent ovulation in animals stimulated to ovulate. In studies carried out in support of the present invention, and described in Example 10, female rats were primed with gonadotropin. Prior to stimulation with hCG, the animals IL-1ra was administered to the animals, and ovulation was significantly inhibited.

### V. Contraceptive Compositions and Methods of Treatment

#### A. Methods of Contraception

##### 1. Prevention of Ovulation and Implantation

The invention includes in one aspect a method of contraception by prevention of implantation of the fertilized ovum or embryo. This aspect of the invention may also include prevention of ovulation. According to this method, an IL-1 receptor antagonist is delivered to the uterus during the time period between coitus and the implantation period; that is, during a period 1-2 days before ovulation or 3-7 days after ovulation. The significance of these time periods will become more apparent with reference to the temporal events depicted in Figure 5, as described below.

IL-1 receptor antagonists for use in preventing ovulation and embryonic implantation are selected as described in Sections II and III, above. That is, suitable IL-1 receptor antagonist compounds are selected for their ability to (i) bind to the IL-1 tI receptor, and/or (ii) antagonize the effects of IL-1 $\alpha$  or IL-1 $\beta$  at the receptor. Such compounds will exhibit a pharmacological profile that is substantially similar to that of IL-1ra or icIL-1ra, and will have very little or no IL-1 agonist



activity. IL-1ra and derivatives thereof may be used in the method of treatment, in accordance with the working examples described in Section IV, above.

Figure 5 shows a temporal depiction of the human menstrual cycle, in which day 1 indicates initiation of menstrual flow. Generally, ovulation occurs at about day 14 of the cycle, as depicted; however, this may vary considerably in and among individual females. In determining the pre-implantation period, it is necessary to refer to the day of ovulation, such a depiction must be taken as a convenient guideline, rather than a standard. For the purposes of determining the peri-implantation period in an individual, the reference point is preferably the day of ovulation. Generally, ovulation occurs just subsequent to a spike of LH and FSH levels in serum. These and other hormonal or physiological measures of ovulation may be measured according to standard methods known in the art. Alternatively, the day of coitus may be used as a reference point, in which case the pre-implantation period will extend to the outer limits shown in Figure 5, as described below. As described above, in humans, sperm are thought to remain viable in the female uterus and fallopian tubes for at least 2 and possibly as long as 5 days following coitus. The ovum is viable for about 24 hours following ovulation. Using the 2 day viability period for sperm, a fertilization period of 3 days (0-3 days post-coitus (p.c.); days 12-15 in the idealized menstrual cycle) is indicated in Figure 5.

Since in humans, implantation generally takes place between about 4 and 7 days after ovulation, it can be appreciated, with reference to Figure 5, that this time will be from about day 3 to about day 11 p.c., assuming that coitus may have occurred at any

time between day 12 and day 15 of the idealized cycle. This implantation period is indicated as days 18-22 in the menstrual cycle. These events, coupled with the animal studies reported in Section IV, define a pre-implantation period that ranges from about day 12 to about day 18 of an idealized menstrual cycle. Preferably and practically, in accordance with the present invention, it will be appreciated that prevention of implantation will be effected by administration of an IL-1 receptor antagonist between about day 3 post-ovulation and about day 7 post-ovulation. When the day of ovulation is uncertain, it will be assumed that it spans the three day fertilization period shown in Figure 5. In such a case, compound will preferably be administered between about day 1 and about day 9 p.c. More preferably, the compound will be administered between about day 3 and day 7 p.c.

In accordance with the invention, it is appreciated that an IL-1 receptor antagonist will also be effective to inhibit ovulation, in the case where ovulation has not occurred prior to intercourse, such as during the period 1-2 days before ovulation (days 12-14 in the idealized cycle illustrated in Figure 5). In such a case, if the time of ovulation is uncertain, the compound will be effective if administered to the subject between about day 0 and day 9 post-coitus.

## 30           2. Dislodging the Implanted Embryo

With continued reference to Figure 5, it will be appreciated that prevention of pregnancy can also be achieved, when IL-1 receptor antagonism interferes with implantation after the initial implantation event. According to the cycle shown in the figure, where implantation occurs between day 18 to day 21 of the cycle, post-implantation

interference, or "dislodgment" will occur as early as day 18 and as late as day 28 in the cycle. Preferably, such dislodgement will be effected between about day 4 and day 10 following ovulation.

5

#### B. Pharmaceutical Compositions

The dosage of IL-1 receptor antagonist required to disrupt implantation will depend on the specific compound and the route of administration, as described below. Generally, an effective dosage will be a dosage that provides in the uterus a concentration that is equivalent to a concentration of IL-1ra of about 1-50  $\mu\text{g/ml}$ . In humans, serum concentrations of about 30  $\mu\text{g/ml}$  have been achieved by administering an intravenous dose of 10 mg/kg IL-1ra (Granowitz).

Although any mode of administration may be used, so long as it provides sufficient concentration of compound to the uterus, it can be appreciated that certain modes of administration will be preferred in the treatment method. In the case of IL-1ra, intravenous administration is possible; alternatively, for convenient self-administration by the patient, other modes of administration, such as nasal insufflation or intravaginal insertion may be preferable. The relative pharmacokinetics of such modes of administration are known in the art.

In one preferred embodiment, the IL-1 receptor antagonist will be formulated in an intravaginal suppository insert having slow release properties, such as are afforded by the formulation described in U.S. Patent No. 5,116,619, incorporated herein by reference. This formulation, which includes, in addition to the active ingredient, about 65-85% lactose, 2-4% starch paste, 2-4% corn starch and 0.4-0.6% magnesium stearate, allows for prolonged

release of active ingredient over 48-72 hours. It can be appreciated that such a suppository formulation can be dispensed for alternate night self-administration, during the peri-implantation  
5 period determined according to the practitioner's understanding of the particular patient's ovulatory cycle and the principles taught herein.

Another preferred administration mode includes the use of a cervical cap to deliver IL-1ra to the  
10 uterus through the cervix. Cervical caps, such as the cervical cap described in U.S. Patent No. 4,961,436 incorporated herein by reference, are known in the art, and may be used to deliver over the peri-implantation period, a medicament  
15 containing an IL-1 receptor antagonist effective to produce in the uterus a concentration of the antagonist equivalent to about 1-50  $\mu\text{g/ml}$  IL-1ra.

In accordance with current techniques in transdermal iontophoresis of peptides, it is also  
20 appreciated that IL-1 receptor antagonist peptides may be administered by transdermal delivery, as through "patch" application. Example 4C. describes a system suitable for transdermal delivery of a peptide formulation suitable for use in the methods  
25 of the present invention.

The following examples illustrate, but in no way are intended to limit the present invention.

#### Materials

30 Interleukin  $1\alpha$  (IL- $1\alpha$ ) and Interleukin  $1\beta$  (IL- $1\beta$ ) were obtained from Genzyme (Norwalk, CT) and are also available from Gibco (Grand Island New York).

All other reagents may be obtained from the suppliers listed in the text or are standard in the  
35 field.

Example 1Localization of IL-1 and IL-1R tI  
in Uterine and Embryonic TissuesA. Culture of Endometrial Cells

5 Human endometrial samples from ovulatory women undergoing hysterectomy for non malignant indications were obtained in the secretory phase. Endometrial stromal cells (ESC) and epithelial cells (EEC) were isolated according to standard  
10 procedures, cultured, and grown in basal conditions in steroid-free media supplemented with 10% serum until confluent monolayers were formed. Endometrial tissues samples were minced into small pieces of less than 1 mm, and subjected to mild collagenase  
15 digestion as follows: Tissue pieces were incubated with shaking for 2 hours at 37°C, in DMEM (Gibco, Grand Island, NY) containing 0.2% collagenase Type I and 0.005% deoxyribonuclease Type I (Worthington, Freehold, NJ). ESC and EEC were isolated from the  
20 mixture by centrifugation, then cultured, and grown to confluence in 75% Dulbecco's Modified Eagle's Medium (Gibco, Grand Island, NY) and 25% MCDB-105 (Sigma, St. Louis, MO), containing antibiotics, supplemented with 10% serum (Charcoal-Dextran  
25 treated FBS, Hyclone, Logan, Utah) and 5 µg/ml insulin (Sigma, St. Louis, MO). ESC and EEC were grown in steroid free medium until they were confluent monolayers. The homogeneity of cultures was determined by morphological characteristics and  
30 verified by immunocytochemical localization of cytokeratin, vimentin, and CD68 antigen as markers for epithelial cells, stromal cells, and human macrophages, respectively. As defined by these criteria, ESC monolayers contained less than 2% of  
35 EEC and less than 0.1% of macrophages. EEC cultures contained less than 3% of ESC and less than 0.1% of macrophages. After cells were confluent (1-2 weeks), the media was supplemented with 5 µg/ml

ascorbic acid, 10 µg/ml transferrin and hormones, as indicated below.

Monolayers were cultured for 6 days with progesterone (P) (10 nM) and epidermal growth factor (EGF) (3.3 nM), according to an established model for *in vitro* decidualization (Irwin). On day 6, human recombinant IL-1b (hrIL-1b) (10 IU/ml), human chorionic gonadotropin (HCG) (10 IU/ml), platelet-activating factor (PAF) (200 nM), or conditioned media from human blastocyst (HBCM; 1ml) were added to the media and cells were further cultured for 2 days and compared with control cells cultured only with P and EGF.

HBCM is conditioned media (500-1000 ml) from human embryos cultured *in vitro* until blastocyst stage. To prepare HBCM, embryos were cultured in Human Tubal Fluid medium (HTF Medium, Irvine Scientific, Santa Ana, CA) containing penicillin (0.5 mg/ml), streptomycin (0.5 mg/ml) and 1% human serum albumin (Fraction V, Irvine Scientific) for approximately 4 to 5 days. The medium covering these cells was then collected, frozen, and stored at -70°C degrees until use. Growth medium was renewed every 2 days, and cells were removed every 2 days for RNA analysis.

#### B. Regulation of IL-1ra Expression by Embryonic Factors

RNA from cultured endometrial cells was analyzed by Northern analysis with human IL-1R tI cRNA probe. A 28S rRNA cDNA probe was used to normalize the data as according to standard methods (13, 14). Data were expressed as arbitrary units:

IL-1R tI mRNA

28S rRNA

Total RNA was extracted from ESC cells according to methods known in the art (Chomczynski),

then 10  $\mu$ g samples were fractionated by electrophoresis according to standard methods (Ausubel) and transferred to a nitrocellulose membrane. The filters were hybridized with a cRNA probe for human IL-1R tI (Simms). Equal loading of RNA on the filters was confirmed by densitometric analysis of 28S rRNA cDNA hybridization signals. IL-1R tI mRNA levels were normalized to 28S rRNA levels and expressed as an arbitrary unit, the IL-1R tI mRNA/28S rRNA level at day 0 of treatment. Densitometric analysis of Northern blots of IL-1 R tI mRNA from different experiments were combined and expressed as the mean  $\pm$  standard deviation (sd). Protein studies were performed by immunocytochemistry using both indirect immunofluorescence and avidin-biotin methods (Simon).

ESC were cultured until confluence in steroid-free conditions as detailed above, RNA extracted, transferred and hybridized with IL-1R tI cRNA probe. Figures 1A and 1B shows Northern blots and a bar graph of quantitation of IL-1R tI mRNA in cultured human endometrial stromal cells (ESC). In the bar graphs combine values obtained from several experiments as indicated by "n". Values are expressed as mean  $\pm$  standard deviation (sd). The positions of 18S and 28S ribosomal RNA are indicated to the left of the Northern blots.

With reference to Figure 1A, lane 1 IL-1R tI mRNA expression at day 0, prior to any further treatment of cells. This lane serves as control. IL-1R tI mRNA levels of ESC treated with progesterone and epidermal growth factor (P+EGF) for 6 and 8 days are shown in lanes 2 and 3, respectively. Lanes 4-7 show blots from ESC treated with P+EGF for 6 days, then, treated for 2 additional days (until day 8) with media from human

blastocyst (1 ml total, Lane 4), 200 nM PAF (Lane 5), IL-1b 10 IU/ml (Lane 6), or hCG 10 IU/ml (Lane 7).

Figures 2A and 2B show the same representations described above for Figures 1A and 1B for endometrial epithelial cells (EEC). EEC cultured until confluence in steroid free-conditions were treated as described for ESC, above. Treatments and lanes correspond to those described above for ESC treatment.

### Example 2

#### Immunodetection of IL-1R tI and IL-1 $\beta$

A female mouse was given 5 IU pregnant mare serum gonadotropin (PMSG) by I.P. injection. Twenty-four hours later, the uterus was removed and stained with rat monoclonal anti-mouse IL-1r TI antibody, according to standard immunohistochemical methods (Simon, et al.) (14). Experiments were repeated with at least three different samples in each case, unless otherwise indicated herein.

Figure 3 shows the results of studies using indirect immunofluorescence to localize of immunoreactive endometrial mouse IL-1R tI (A-D) and immunoreactive IL-1 $\beta$  (E) in the mouse embryo. Figure 3 (A-E) shows results (A) Mouse uterus obtained 24 h. after pregnant mare serum gonadotropin (PMSG) injection (5 IU); mouse IL-1R tI is localized in the apical region of the luminal epithelium and in scattered areas of the stroma; (original magnification x400). (B) Mouse uterus obtained 24 h. after hCG injection (5 IU); Stronger IL-1R tI staining throughout the epithelium and in the stroma is observed; (x400). (C) Negative control by deletion of the primary antibody; (x400). (D) Mouse uterus obtained at day 4 of pregnancy (single experiment). Increased staining was observed for



IL-1R tI in the epithelium surrounding the blastocyst (arrow); (x400).

Figure 3E shows immunolocalization of mouse embryonic IL-1b, at day 9 of pregnancy with twin embryos (indicated at "e" in the figure) immunoreactive IL-1b is present in the mouse placenta (indicated at "p" in the figure) with increased staining at the interface with the maternal endometrium (indicated at "m" in the figure); (x400). Monoclonal antibodies against mouse IL-1b and the extracellular domain of the mouse IL-1R tI were acquired from Genzyme (Cambridge, MA).

### Example 3

#### 15      Inhibition of Embryonic Implantation in vivo

##### A.      Periodic Injection of Il-1ra

Female 12-week-old B6C3F-1 female mice (approximately 20 g) were stimulated with 5 I.U. of Pregnant Mare's Serum Gonadotropin (PMSG; Sigma, St. Louis, MO) i.p., followed 24 hours later by 5 I.U. human chorionic gonadotropin (hCG) i.p. administration. The mice were then mated immediately with males of the same strain and age. Females with vaginal plugs (designated pregnancy day 1) were randomly allocated to three groups: A, Control non-injected; B, Buffer injected animals and C, animals injected i.p. with 20 µg of recombinant human IL-1ra (rh IL-1ra) every 12 hours beginning on pregnancy day 3 and ending on day 9. Intraperitoneal injections were carefully directed away from the pregnant uterine horns. Injections were continued until day 9 to avoid the possibility of delayed implantation, and animals were sacrificed 12 hours after the last injection. Animals appeared healthy throughout the experiment. Implantation sites in both uterine horns in IL-1ra injected animals were counted and compared with control

animals receiving buffer or no injection. Two different preparations of IL-1ra (26) and two different buffers were used in four separate experiments. Data were analyzed by Fisher Exact  
5 Test. Results of these studies are shown in Table 1, in Section IV.C.3, above.

B. Morphological Assessment of Effects of IL-1ra on Embryonic Implantation

10 PMSG/hCG stimulated 12-week-old B6C3F-1 females were mated with males of the same strain and age. Females with vaginal plugs (designated pregnancy day 1) were randomly allocated to two groups: I, Buffer injected animals, and II, animals  
15 injected i.p. with 20  $\mu$ g of recombinant human IL-1ra (rh IL-1ra) (26) every 12 hours beginning on pregnancy day 3 until day 9. Starting on day 3, one mouse from each group was sacrificed each day. Uteri were removed and immediately fixed in 10%  
20 formalin in phosphate buffered saline. Fixed tissue were embedded in paraffin, sectioned and mounted on glass slides. Serial sections (4  $\mu$ m) from each sample were then stained with hematoxylin-eosin (H&E) and viewed with an Olympus 35 mm camera and an  
25 Olympus BH2 microscope.

Results are shown in Figure 4 (A-F). Micrographs from untreated animals are shown in Figures 4A, 4C, and 4E. Human recombinant IL-1ra treated animals are shown in figures 4B, 4D, and 4F.  
30 Figure 4A shows day 4 normal pregnancy; longitudinal section of a free blastocyst surrounded by enlarged uterine luminal epithelial cells [Hematoxylin/Eosin (H & E), original magnification x400]. Figure 4B shows Day 4 pregnant animals injected with IL-1ra;  
35 Cross-section of a free blastocyst. In this micrograph, the epithelium surrounding the blastocyst is not enlarged as observed with normal pregnancy (H & E x400). Figure 4C shows the uterus

of day 7 normal pregnant mouse; longitudinal section through embryo and decidual mass demonstrating complete stromal implantation (H & E x400). Figure 4D shows the uterus of day 7 of pregnant mouse, IL-1ra injected. Here the blastocysts are still free. No attachment or invasion is observed (H & E x400). Figure 4E shows the uterus of day 9 normal pregnant mouse; longitudinal section through embryo and decidual mass (H & E x200). Figure 4F shows the uterus of day 9 of pregnancy, IL-1ra injected mouse; stromal decidual reaction (arrows) with intact glandular epithelium and degenerated trophoblastic cells in the uterine lumen (arrowhead) can be observed, but implantation did not occur in this animal (H & E x800). This is a section from the single IL-1ra injected mouse described in Table 1 that was macroscopically pregnant.

C. Transdermal delivery of IL-1 receptor antagonist peptides

Female rodent subjects, preferably hairless guinea pigs or mice (subjected to a depilatory agent) are mated, and examined for evidence of pregnancy as described in Part A, above. Animals are then fitted with a transdermal patch system, according to methods described by Kumar, et al. (J. Controlled Release 18: 213-220, 1992), incorporated herein by reference. Briefly, the patch consists of a die cut reservoir ring of closed cell polyethylene foam having contact adhesive on one side. The ring is fitted one side with a Millipore GVWP hydrophilic filter. The ring and the filter join to form an anode reservoir, into which is placed a gel containing IL-1 receptor antagonist. The gel consists of Polyjel HV (polyglyceryl citrate + hydroxypropyl methyl cellulose; Guardian Chemical, Hauppauge, NY) mixed with acetate buffer (0.05 M, pH

5.8) into which is mixed the peptide at about 0.1% (wt/wt). The reservoir is closed with a transparent polyethylene film having contact adhesive on one side, into which is embedded a stainless steel snap button electrode. Alternatively, for use with acidic molecules, a cathode patch, consisting of a karaya gumpad (Iomed, Inc., Salt Lake City, UT) is used.

The patch is connected to a pulsed voltage output power source with a current limiting feature.

Animals are treated using a current limit of 0.17 mA/cm<sup>2</sup> at 50 KHz and 50% duty cycle or 0.85 mA for a total area of 5 cm<sup>2</sup>, for 5 hours. Blood is sampled for presence of the peptide, to determine the delivered dose. Control animals are given the carrier gel, without IL-1 receptor antagonist compound.

After an appropriate gestational period, as described in part A above, animals are then tested for presence implanted embryos, as described in Example 3, part A, above.

#### Example 4

##### Effect of IL-1ra on Embryonic Development

##### A. Culture of Early Embryos with IL-1ra

2-cell mouse embryos were flushed from the oviducts on day 1 of pregnancy from animals impregnated as described in Example 3. Embryos (n=276) were rinsed and placed in 4-well Nuncon plates containing 500  $\mu$ l of human tubal fluid medium (HTF Medium, Irvine Scientific Santa Ana, CA) with 0  $\mu$ g/ml (n=91), 1  $\mu$ g/ml (n=36), 50  $\mu$ g/ml (n=36), 100  $\mu$ g/ml (n=52), and 200  $\mu$ g/ml (n= 61) of rh IL-1ra. After 72 h in culture the percentage of embryos reaching the blastocyst stage was 85.7%, 91.6%, 94.4%, 96% and 85.2% respectively .

B. Effects of IL-1ra on Blastocyst Development

Effects of rh IL-1ra on blastocyst hatching, trophoblast outgrowth and migration, were determined by further culture of the blastocysts for 5 days on fibronectin-coated plates (Upstate Biotechnology, Inc., Lake Placid, NY, 27). Ten blastocysts obtained from control wells (0  $\mu\text{g/ml}$  rh IL-1ra) and 10 from wells grown in the presence of 200  $\mu\text{g/ml}$  of rh IL-1ra were placed in plates containing 200  $\mu\text{g/ml}$  of rh IL-1ra. Ten blastocysts from control and IL-1ra containing cultures were placed in control plates lacking IL-1ra. In both groups, hatching, outgrowth and migration were documented to be similar in the presence or absence of rh IL-1ra.

Example 5

Recombinant Expression of IL-1ra

Plasmids containing the human IL-1 receptor antagonist (hIL-1ra) gene were obtained Immunex Corporation, Seattle, WA. The gene sequence of the insert is shown as SEQ ID NO: 1 in Figure 6 herein. Alternatively, the gene can be synthesized using an oligonucleotide synthesizer, or obtained by the isolation/synthetic method described in the reference by Carter, et al., incorporated herein by reference, and verified, using the sequence shown as SEQ ID NO: 1. Alternatively, primers based on this sequence can be used for preparation of the gene sequence, according to standard procedures (Ausubel).

For use in experiments carried out in support of the present invention, recombinant *E. coli* human IL-1ra was expressed as a fusion protein using the pMAL vector (New England Biolabs). The expressed fusion protein was detected and purified from the periplasm of *E. coli* by Amylose affinity chromatography (New England Biolabs). The amylose

eluate pool was dialyzed into 50 mM Tris-HCL, 100 mM NaCl pH 7.5 and cleaved with Factor Xa (Enzyme Research Labs) at a concentration of 10 ng to 50  $\mu$ g of fusion protein. SDS-PAGE was run to detect Factor Xa cleavage of the fusion protein. Homogeneity of hIL-1ra was achieved with further purification onto Q-Sepharose pH 8.5 and Sephacryl S-300 gel filtration chromatographies (Pharmacia). The purified protein has a molecular mass (Mr) of 17,000 dalton. Human IL-1ra activity was measured by a mouse D10 cell proliferation assay, protein quantitation by gel laser densitometer (Molecular Dynamics) and endotoxin level was measured by the Limulus assay (Whittaker Bioproducts).

15

#### Example 6

##### Purification of IL-1ra

IL-1ra is purified from cellular sources as described by Carter, et al. as follows: U937 cell cultures are obtained from the American Type Culture Collection, Rockville, MD. Cells are cultured at 37°C for 48 h at  $5 \times 10^5$  cells  $\text{ml}^{-1}$  in RPMI 1640 medium (GIBCO), 7% fetal bovine serum (FBS, GIBCO), 2mM L-glutamine, 100 U  $\text{ml}^{-1}$  penicillin, 100  $\mu$ g  $\text{ml}^{-1}$  streptomycin, 20mM HEPES buffer, and 100 nM phorbol myristate acetate (PMA) (Sigma, St. Louis, MO). After differentiation, the medium is removed and the cells, now adherent, are gently washed once with a small volume of Dulbecco's PBS. Cells are then cultured for an additional 48 h in RPMI 1640 containing 1% low-endotoxin FBS (HyClone, Logan, UT), L-glutamine, penicillin, streptomycin, and rhGM-CSF (75 U  $\text{ml}^{-1}$ ; Amgen, Torrance, CA). Cell-free supernatants are collected, pooled and frozen at -20°C. After thawing, phenylmethylsulphonyl fluoride is added to 0.2 mM final concentration. The solutions are concentrated by ultrafiltration with

35

YM-5 and YM-10 filters (Amicon). Protein measurements are made using Bradford assay (Bio-Rad Kit) using BSA as the standard protein.

The preparation is then subjected to Superose 12 FPLC-1 chromatography in 17 repetitive cycles. Two-ml aliquots of Fraction 2 concentrate are injected onto a Superose 12 prep grade FPLC column (1.6 x 50 cm) equilibrated with 0.1 M potassium phosphate, 1 M NaCl, pH6.0. Fractions of 1 ml are collected at a flow rate of 1 ml min<sup>-1</sup>. Fractions are collected and individually concentrated by Centricon-10 units (Amicon). After analysis by SDS-PAGE and bioassay for IL-1ra activity, appropriate fraction concentrates are pooled and further concentrated to 1 ml by Speed Vac centrifugation (Savant).

Pooled samples are then subjected to TSK Bio-Sil 125 HPLC-1, as follows: in 20 repetitive cycles, 50 µl of fraction 3 are injected onto a TSK-Bio-Sil-125 HPLC column (7.5 x 600 mm), using the same buffer system as described above. Fractions of 200 µl are collected. Appropriate fractions are pooled and concentrated by Centricon-10 filter units and finally by Speed Vac centrifugation as described above.

Pooled samples are further purified, using TSK Bio-Sil 125 HPL-2, as follows: in seven repetitive cycles, 20 µl of fraction 4 are injected onto the same column as described above. Active fractions are pooled to give a final volume of 8.5 ml, and are subjected to C4 reverse phase HPLC, as follows: appropriate fractions from the TSK Bio-Sil 125 run (fraction 5) are injected directly onto a C4 reverse phase column (4.6 x 150 mm). Proteins which remain bound to the matrix following an extensive wash with 0.1% trifluoroacetic acid (TFA) are desorbed using a linear gradient from 28 to 32% acetonitrile in

0.1% TFA over a period of 44 min at a flow rate of 1 ml min<sup>-1</sup> (room temperature). Representative aliquots of each fraction or pool are removed, taken to complete dryness by Speed Vac centrifugation, and  
5 then re-solubilized in tissue culture medium in preparation for assay.

#### Example 7

##### IL-1 receptor binding assays

10 Radioiodinated IL-1 $\alpha$  and IL-1 $\beta$  are obtained from commercial sources (DuPont NEN Research Products, Boston, MA) or prepared by chloramine-T radioiodination of unlabeled peptide.

EL-4 cells (6x10<sup>6</sup>/ml; mouse lymphoma cells; ATCC  
15 TIB 39, American Type Culture Collection, Rockville, Maryland) are suspended in binding medium (RPMI 1640 containing 5% fetal calf serum and 25 mM HEPES, pH 7.2). Aliquots (100  $\mu$ l) are placed in 12 x 75 mm polypropylene tubes. <sup>125</sup>I-labelled IL-1 is added at  
20 a concentration of about 1 x10<sup>-10</sup> M. Incubation is carried out for 3 hours at 4°C in the presence of test compound, as described below. Nonspecific binding is determined by inclusion of 1 x10<sup>-8</sup> M unlabeled IL-1. Unbound <sup>125</sup>I-IL-1 is separated from  
25 cells by centrifugation through 200  $\mu$ l of a silicone oil mixture in a Beckman microfuge.

Displacement of binding by test compound is determined by adding to the above incubation mixture, varying concentrations of unlabeled test  
30 compound ranging from about 10<sup>-12</sup>-10<sup>-8</sup> M compound. IC<sub>50</sub> and K<sub>i</sub> values are calculated for the test compound according to methods well known in the art (Kenakin).



Example 8Inhibition of Cellular ProliferationA. Inhibition of IL-1 stimulation of mouse D10 cell

5        Mouse T helper D10.G4.1 cells (American Type Culture Collection, Rockland, MD; ATCC TIB 224) were suspended in culture medium (RPMI 1640 containing 5% fetal calf serum,  $5 \times 10^{-8}$  M 2-mercaptoethanol, 8  $\mu\text{g/ml}$  gentamicin, 2 mM L-glutamine, and 2.5  $\mu\text{g/ml}$  concanavalin A). Cells were suspended to produce a  
10       cell concentration of about  $1-2 \times 10^6 \mu\text{g/ml}$ . IL-1ra was added to triplicate cultures, 1 hour prior to addition of IL-1 $\alpha$  or IL-1 $\beta$  to the cultures. The plates were then incubated and incorporation  
15       of [ $^3\text{H}$ ]Thymidine into the cells measured according to standard techniques. In experiments carried out in support of the present invention, hIL-1 $\alpha$  or hIL-1 $\beta$  was added in increasing concentrations to the assay, the  $\text{EC}_{50}$  of hIL-1 $\alpha$  was 7 pM and the  $\text{EC}_{50}$  of hIL-1 $\beta$  was  
20       450 pM. At a concentration of 30 nM, hIL-1ra completely inhibited stimulation of proliferation by hIL-1 $\alpha$  to a concentration of 100 pM and by hIL-1 $\beta$  to a concentration of about 100 nM.

      Determination of  $\text{IC}_{50}$  and  $\text{K}_\text{D}$  values for IL-1  
25       receptor antagonists in these assays is accomplished according to methods known in the art.

B. Inhibition of IL-1 Stimulation of Thymocytes

      Mouse thymocytes are prepared, and the action  
30       of IL-1, which produces growth stimulation, is measured using standard techniques (O'Gara, 1990). Three to six week old C3H/HeN mice are obtained from Simonsen Laboratories, Gilroy, California and sacrificed by standard procedures. Thymi are  
35       immediately removed, separated from adherent non-thymic tissue, homogenized in Hank's balanced salt solution (Gibco) using a glass homogenizer, and

centrifuged at 180 x g for 10 minutes at 15°C. Following an additional wash in HBSS, the thymocytes are resuspended in RPMI 1640 tissue culture medium (Gibco) containing 50 $\mu$ M 2-mercaptoethanol (Fisher),  
5 2 mM glutamine (Gibco), 1 mM pyruvate, non-essential amino acid solution, penicillin (100 U/ml) streptomycin (100 $\mu$ g/ml) solution, 10% heat inactivated fetal bovine serum and Phytohemagglutinin (PHA, Pharmacia, final concentration 10  $\mu$ g/ml).  
10 Cells are cultured in round-bottom 96 well microtiter tissue culture plates, 6x10<sup>5</sup> cells per well in a volume of 100  $\mu$ l. Test compound is diluted in tissue culture medium and added to the wells in the presence and absence of IL-1  
15 (recombinant human IL-1, R&D Systems catalog # 201-LB, 0.1 ng/ml). Total volume is 150  $\mu$ l per well. Plates are incubated for 72 hours (95% air/5% CO<sub>2</sub>, 37°C). During the last four hours of incubation, tritiated thymidine (Amersham, 49 Ci/mM)  
20 is added (0.5  $\mu$ Ci per well). Cells are harvested onto Whatman 934-AH glass microfiber filters and counted in a Beckman LS 6000 scintillation counter. Results are expressed as counts per minute well. Cytotoxicity is measured in these assays using the  
25 MTT reduction assay, as described below.

Untreated cells show minimal DNA synthesis (thymidine incorporation 80 cpm/well). PHA alone produces a 2-3 fold stimulation of cell proliferation. Treatment with 0.1 ng/ml IL-1 in the  
30 presence of PHA results in 60 fold stimulation. Addition of the IL-1ra results in a dose dependent inhibition of IL-1 stimulation.

#### Example 9

##### 35 IL-6 Bioassay

Mice were given IL-1 are injected with IL-1ra (5 mg/kg, subcutaneously, (s.c.)) or test receptor

antagonist. Mice are immediately given IL-1 $\alpha$  (5  $\mu$ g/kg s.c.). Blood samples from the mice are taken 3 hours later.

Serum levels of IL-6 are determined using a  
5 modification of the B9 hybridoma cell assay described by Aarden et al. and incorporated herein by reference. B9 cells are treated with twofold serial dilutions of the test sera in 96-well microtiter plates. After a 3-day incubation at 37°  
10 in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air, the wells are pulsed with 0.5  $\mu$ Ci of [<sup>3</sup>H]thymidine ([<sup>3</sup>H]TdR) and incubated for an additional 18 hours. The cells are then harvested onto glass fiber filters and the level of [<sup>3</sup>H]TdR incorporation  
15 determined in a liquid scintillation counter. IL-6 units are defined as the inverse of the serum dilution that produces half-maximal [<sup>3</sup>H]TdR incorporation compared with a reference standard.

20

#### Example 10

##### Prevention of Ovulation

Female rats were administered PMSG (25 I.U.), then anesthetized with pentobarbital. One horn of the uterus was exposed, and 1  $\mu$ g of IL-1ra was  
25 injected unilaterally directly into the ovarian bursa. Animals were then stimulated by administration of hCG (25 I.U.). Animals were later examined for presence of ova. Using the contralateral uninjected ovary as control, a 40%  
30 reduction in ovulation was observed after IL-1ra treatment.

While the invention has been described with reference to specific methods and embodiments, it will be appreciated that various modifications and  
35 changes may be made without departing from the invention.

## IT IS CLAIMED:

1. A composition for use in preventing ovulation and implantation of an embryo in a uterus  
5 of a mammalian subject, comprising an IL-1 receptor antagonist.

2. The composition of claim 1, wherein the interleukin-1 receptor antagonist is a peptide.  
10

3. The composition of claim 2, wherein said interleukin receptor antagonist is selected from the group consisting of IL-1ra (SEQ ID NO: 2) and icIL-1ra (SEQ ID NO: 4).  
15

4. The composition of claim 3, wherein said interleukin receptor antagonist is IL-1ra (SEQ ID NO: 2).

20 5. The composition of claim 1, wherein said compound is selected from a combinatorial library of compounds, by the steps of:

measuring (i) agonist activity, and (ii) antagonist activity of a library of compounds, in a  
25 functional IL-1 receptor assay, and

selecting a specific compound in the library if it has (i) substantially no agonist activity, and (ii) substantial antagonist activity.

30 6. A contraceptive device for use in preventing ovulation and implantation of an embryo or in dislodging an embryo in a mammalian uterus, comprising

a vaginal insert or a cervical cap, and  
35 compound release means in said insert or cap for releasing an IL-1 receptor antagonist compound

at a dose effective to prevent or disrupt implantation of an embryo in the uterus.

7. The device of claim 6, wherein the  
5 interleukin-1 receptor antagonist is a peptide.

8. The device of claim 7, wherein said  
interleukin receptor antagonist is selected from the  
group consisting of IL-1ra (SEQ ID NO: 2) and icIL-  
10 1ra (SEQ ID NO: 4), and said compound release means  
is effective to produce a concentration of said  
peptide at the uterus of about 1-50  $\mu\text{g/ml}$ .

9. The device of claim 6, wherein the said  
15 interleukin receptor antagonist is selected from a  
combinatorial library of compounds, by the steps of:  
measuring (i) agonist activity, and (ii)  
antagonist activity of a library of compounds, in a  
functional IL-1 receptor assay, and  
20 selecting a specific compound in the library if  
it has (i) substantially no agonist activity, and  
(ii) substantial antagonist activity.

10. A method of identifying a compound for use  
25 in preventing ovulation and implantation of an  
embryo or in dislodging an embryo in a mammalian  
uterus, comprising

measuring (i) agonist activity, and (ii)  
antagonist activity of a library of compounds, in a  
30 functional IL-1 receptor assay, and

selecting a specific compound in the library if  
it has (i) substantially no agonist activity, and  
(ii) substantial antagonist activity.

35 11. The method of claim 10, wherein said  
measuring further includes measuring the binding  
affinity of the compound in an IL-1R  $\pm$  IL-1 ligand

competitive displacement binding assay, and said selecting further includes selecting the compound if its affinity in such binding assay is at least within two orders of magnitude of the binding  
5 affinity of an IL-1 receptor antagonist selected from the group consisting of IL-1ra and icIL-1ra.

1/7

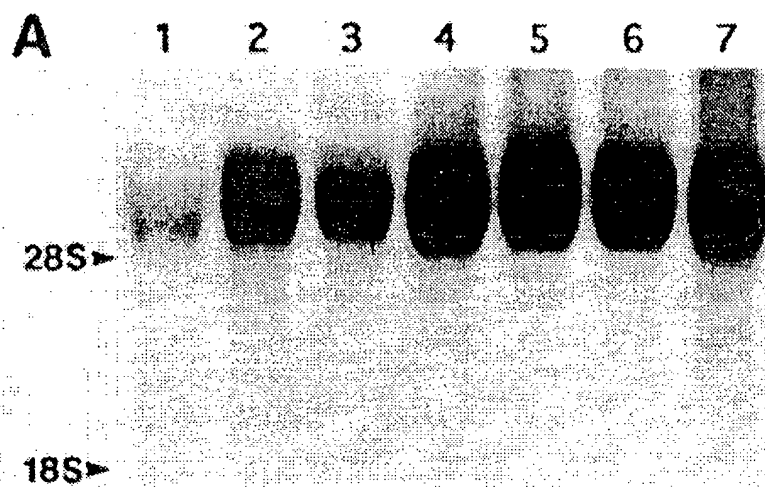


Fig. 1A

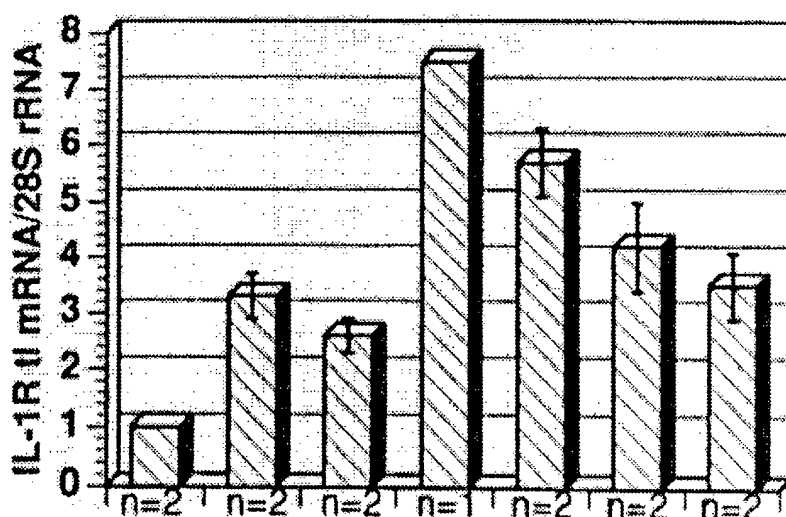


Fig. 1B

2/7

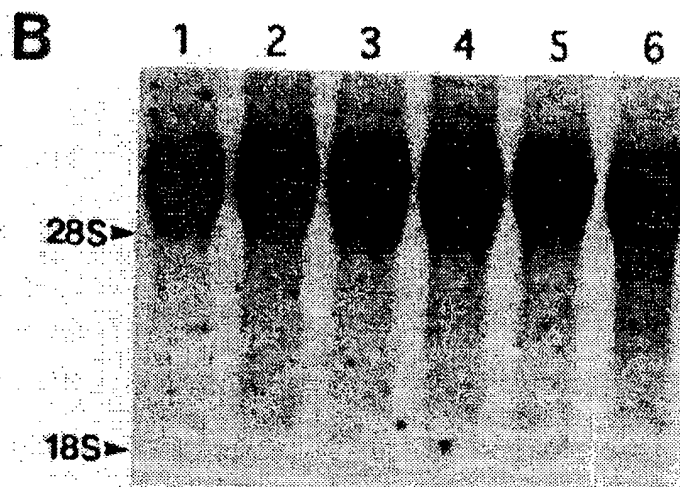


Fig. 2A

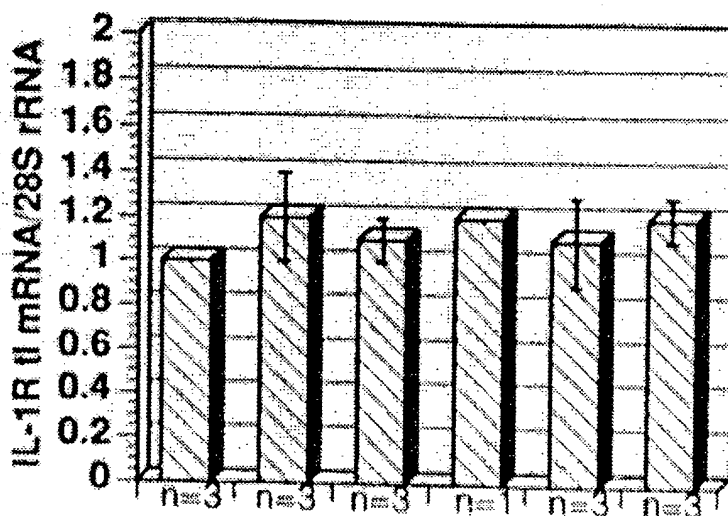


Fig. 2B



3/7



Fig. 3A

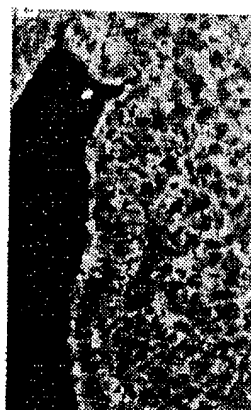


Fig. 3B



Fig. 3C



Fig. 3D

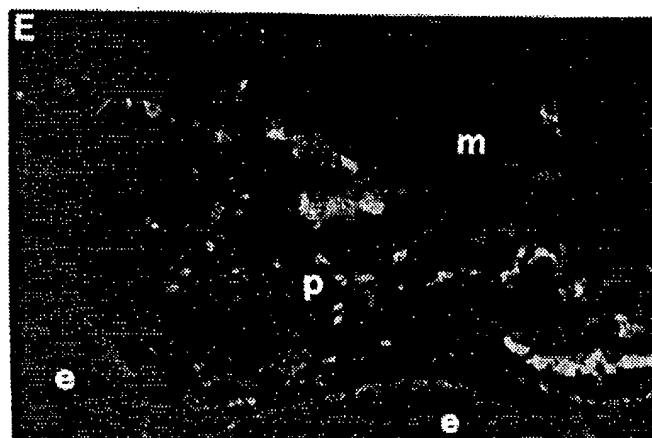


Fig. 3E

4/7

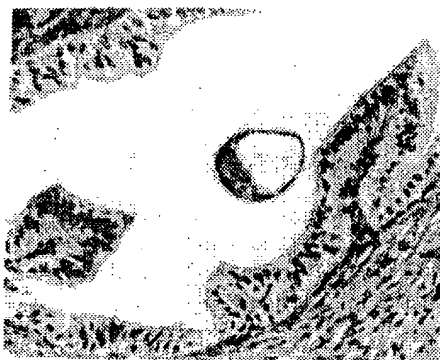


Fig. 4A

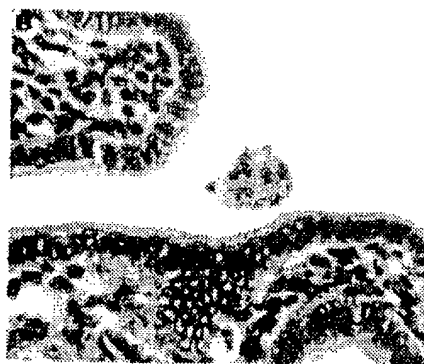


Fig. 4B

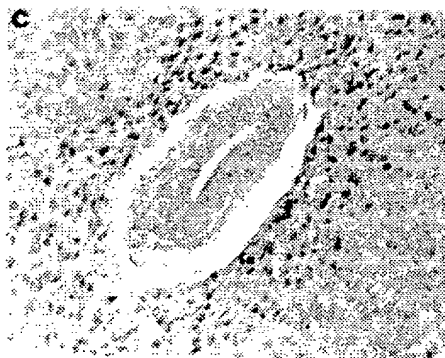


Fig. 4C

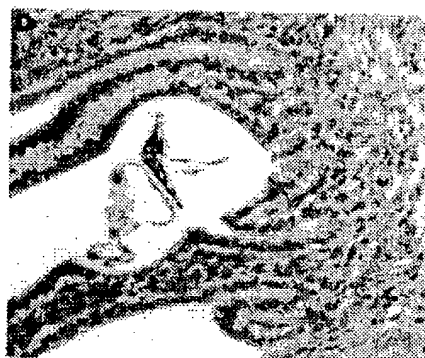


Fig. 4D

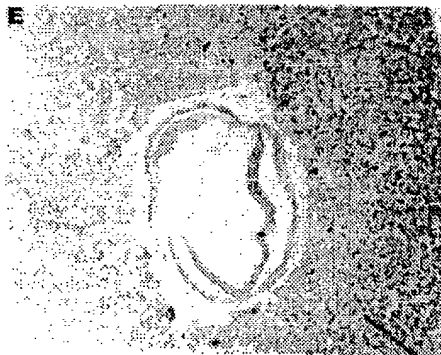


Fig. 4E

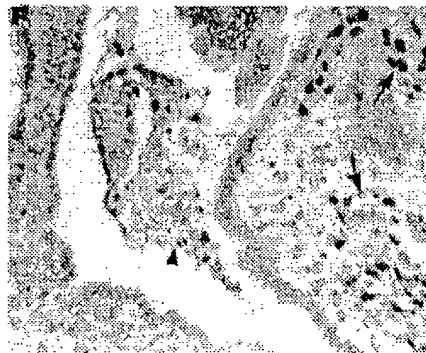
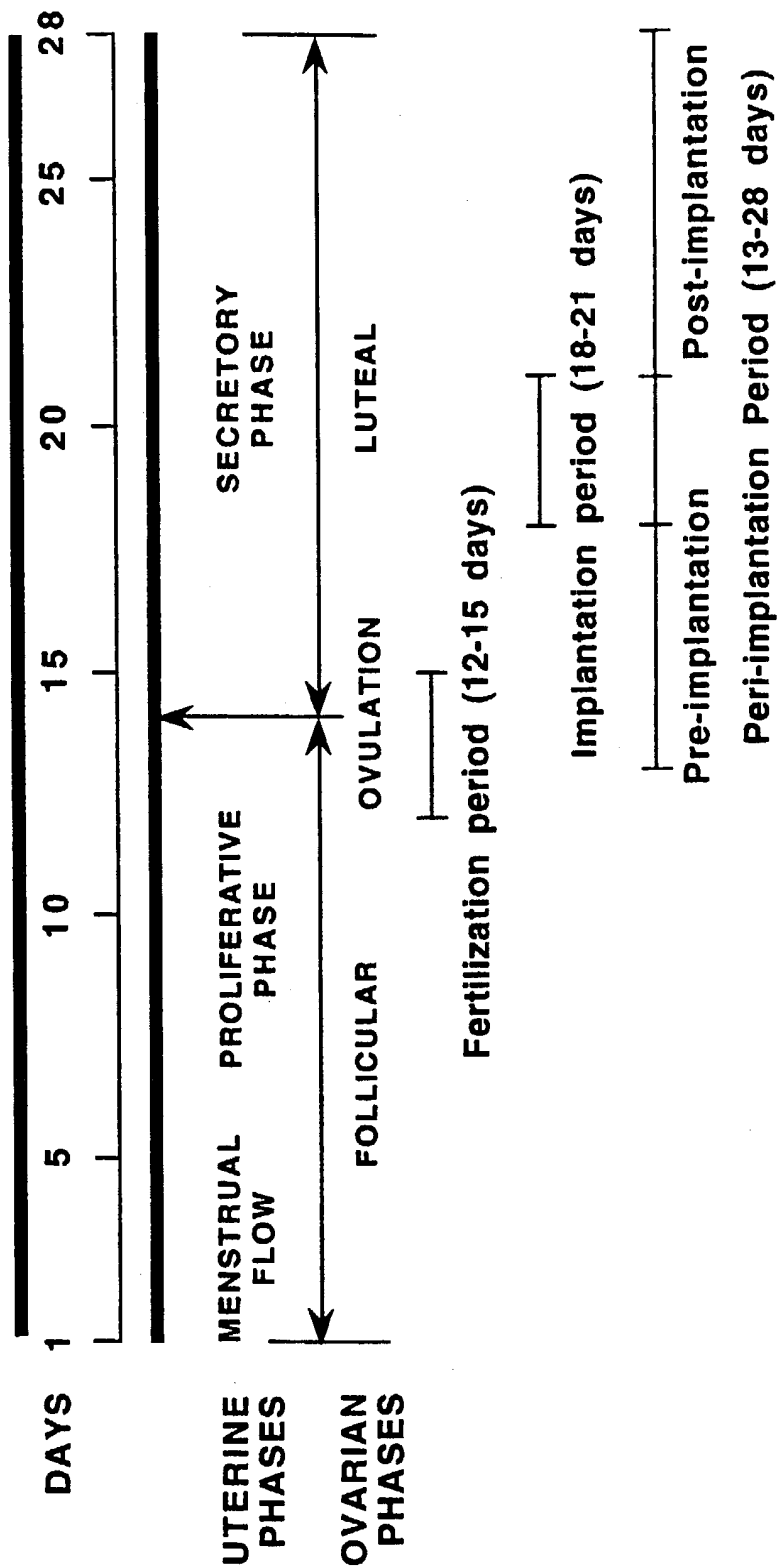


Fig. 4F

Fig. 5



ATG	GAA	ATC	TGC	AGA	GGC	CTC	CGC	AGT	CAC	CTA	ATC	ACT	CTC	CTC	CTC	TTC	CTG	TTC	CAT	23
M	E	I	C	R	G	L	R	S	H	L	I	T	L	L	L	F	L	F	H	83
TCA	GAG	ACG	ATC	TGC	CGA	CCC	TCT	GGG	AGA	AAA	TCC	AGC	CAA	ATG	AAG	GCC	TTC	AGA	ATC	143
S	E	T	I	C	R	P	S	G	R	K	S	S	Q	M	K	A	F	R	I <sub>15</sub>	
TGG	GAT	GTT	AAC	CAG	AAG	ACC	TTC	TAT	CTG	AGG	AAC	AAC	GTT	CTA	CAA	GCT	GGA	TAC	TTG	203
W	D	V	N	Q	K	T	F	Y	L	R	N	N	V	L	Q	A	G	Y	L <sub>35</sub>	
CAA	GGA	CCA	AAT	GTC	AAT	TTA	GAA	GAA	AAG	ATA	GAT	GTG	ATT	CCC	GTA	GAG	CCT	CAT	GCT	263
Q	G	P	N	V	N	L	E	E	K	I	D	V	I	P	V	E	P	H	A <sub>55</sub>	
CTG	TTC	TTG	GGA	ATC	CAT	GGA	GGG	AAG	ATG	TGC	CTG	TCC	AAG	GTC	TGT	TCT	GGT	GAT	GAG	323
L	F	L	G	I	H	G	G	K	M	C	L	S	K	V	C	S	G	D	E <sub>75</sub>	6/7
ACC	AGA	CTC	CAG	CTG	GAG	GCA	GTT	AAC	ATC	ACT	GAC	CTG	AAC	GAG	AGC	AGA	AAG	CAG	GAC	383
T	R	L	Q	L	E	A	V	N	I	T	D	L	N	E	S	R	K	Q	D <sub>95</sub>	
AAG	CGC	TTC	GCC	TTC	ATC	CGC	TCA	GAC	AGT	GGC	CCC	ACC	TTT	AGT	ACC	GAG	TCT	GCC	GCC	443
K	R	F	A	F	I	R	S	D	S	G	P	T	F	S	T	E	S	A	A <sub>115</sub>	
TGC	CCC	GGT	TGG	TTC	CTC	TGC	ACA	GCG	ATG	GAA	GCT	GAC	GTC	CCC	CAG	AGC	CTC	ACC	AAT	503
C	P	G	W	F	L	C	T	A	M	E	A	D	V	P	Q	S	L	T	N <sub>135</sub>	
ATG	CCT	GAC	GAA	GGC	GTC	ATG	GTC	ACC	AAA	TTC	TAC	TTC	GAC	GAG	CAG	GAG	TAG	557		
M	P	D	E	G	V	M	V	T	K	F	Y	F	D	E	Q	E <sub>152</sub>	+			

Fig. 6

7/7

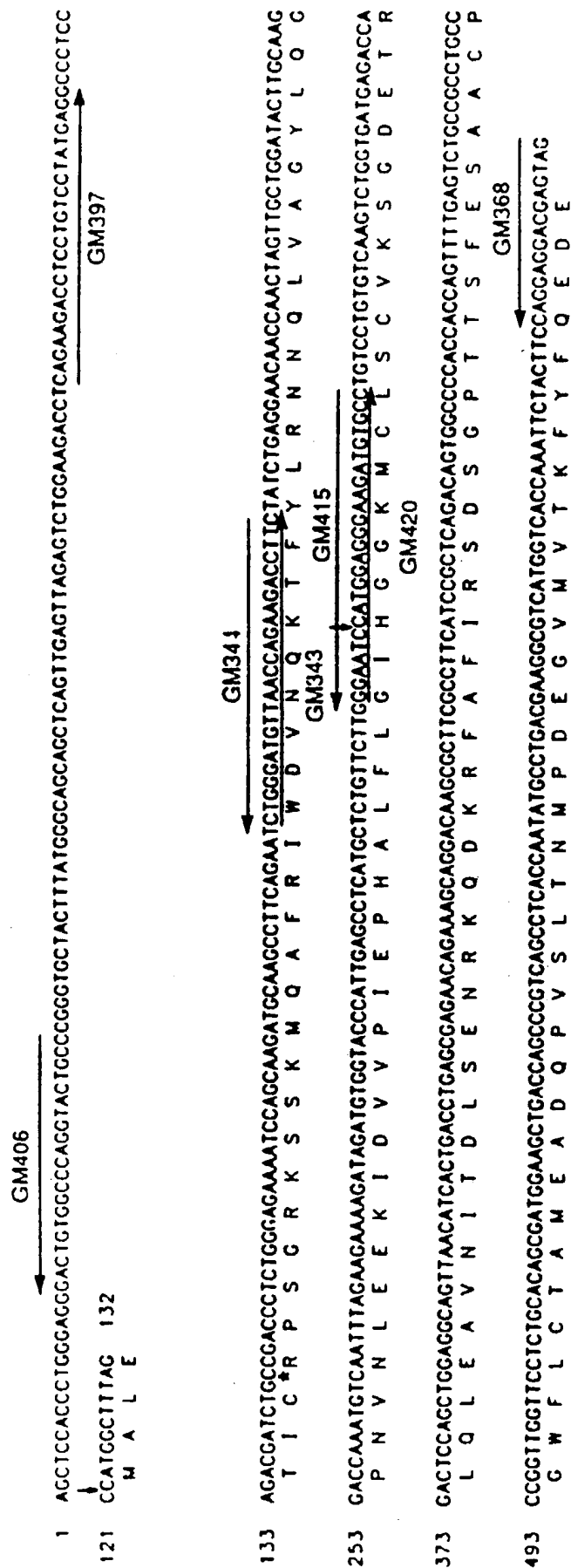


Fig. 7

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/11588

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : A61K 38/20

US CL : 514/12

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

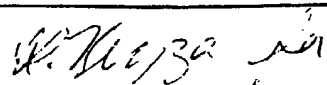
APS, CAS ONLINE, EMBASE, MEDLINE, WPIDS.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proc. Natl. Acad. Sci. USA, Volume 88, issued May 1991, S. Haskill et al., "cDNA cloning of an Intracellular Form of the Human Interleukin 1 Receptor Antagonist associated with Epithelium", pages 3681-3685, see whole document.	1-2, 5-7, 9-10
Y	Biology of Reproduction, Volume 14, issued 1976, I. Aref et al., "Utero-Oviductal Motility, Egg Transport and Implantation in Prostaglandin Treated Rabbits", pages 658-664, see whole document.	1-2, 5-7, 9-10
Y	Am. J. Obstet. Gynecol., Volume 167, No. 4, Part 1, issued October 1992, R. Romero et al., "The natural interleukin-1 receptor antagonist in term and preterm parturition", pages 863-872, see whole document.	1-2, 5-7, 9-10

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 05 DECEMBER 1994	Date of mailing of the international search report FEB 1 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer C. SAYALA  Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/11588

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,116,619 (GRECO ET AL.) 26 May 1992, see whole document.	6-7, 9
Y	US, A, 4,961,436 (KOCH) 09 October 1990, see whole document.	6-7, 9
Y	Nature, Volume 343, issued 25 January 1990, C. Hannum et al., "Interleukin-1 receptor antagonist activity of a human interleukin-1 inhibitor", pages 336-340, see whole document.	5, 9-10
Y	J. Exp. Med., Volume 173, issued April 1991, K. McIntyre et al., "Inhibition of Interleukin 1 (IL-1) Binding and Bioactivity In Vitro Modulation of Acute Inflammation In Vivo by IL-1 Receptor Antagonist and Anti-IL-1 Receptor Monoclonal Antibody", pages 931-939, see whole document.	5, 9-10
Y	J. Immunology, Volume 138, No. 9, issued 01 May 1987, S. Mizel et al., "The interleukin 1 receptor. Dynamics of Interleukin 1 Binding and Internalization in T Cells and Fibroblasts", pages 2906-2912, see whole document.	5, 9-10